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INDUCTION OF OOCYTE SHEDDING AND MEIOTIC MATURATION IN *PISASTER OCHRACEUS:* KINETIC ASPECTS OF RADIAL NERVE FACTOR AND OVARIAN FACTOR INDUCED CHANGES [1]

ALLEN W. SCHUETZ

Division of Population Dynamics, The Johns Hopkins School of Hygiene, 615 North Wolfe Street, Baltimore, Maryland 21205 and Friday Harbor Laboratories, Friday Harbor, Washington 98250

During the process of oogenesis in most animals the breakdown or dissolution of the nucleus (germinal vesicle) is a necessary prerequisite before meiotic maturation can occur. In starfish the induction of oocyte meiotic maturation is closely synchronized with the process of oocyte shedding from the ovary. Both events follow the exposure of ovarian tissue *in vivo* or *in vitro* to a radial nerve factor (RNF) extracted from the radial nerves (Chaet and McConnaughy, 1959; Kanatani, 1964; Chaet, 1966). Although these data indicate that radial nerve factor (RNF) acts on the gonads, it is not clear whether RNF acts directly or indirectly on the oocytes to produce shedding and meiotic maturation. Experiments by Kanatani (1964) indicated that RNF could act directly on oocytes of *Asterias amurensis*, maintained in calcium free sea water, to produce meiotic maturation. In recent studies with *Asterias forbesi* (Schuetz and Biggers, 1967; Schuetz, 1969) and *Asterina pectinifera* (Kanatani and Shirai, 1967), it was demonstrated that the RNF may act indirectly to initiate meiotic maturation. These studies showed that following *in vitro* incubation of ovarian tissue with RNF, the incubation medium, but not RNF, was capable of initiating oocyte meiotic maturation in isolated oocytes. A biologically active substance present in the incubation medium could be physically separated from the RNF. This substance has been called an ovarian factor (Schuetz and Biggers, 1967). Although the RNF and ovarian factor appeared to have different chemical properties their biological properties were remarkably similar. The so-called ovarian factor was shown to: (1) induce oocyte shedding and meiotic maturation in isolated oocytes as well as initiate meiotic maturation in oocytes retained within ligated ovarian fragments, and (2) initiate follicular cell dispersion from around isolated oocytes or oocytes retained within ovarian tissue. Such oocytes were found to be fertilizable and capable of undergoing normal development. These data suggested that this ovarian factor may be a mediator for RNF activities. Data on the time and sequence of changes which occur in oocytes and ovarian tissues following exposure to ovarian factor and RNF could provide support for this hypothesis.

The experiments reported here were conducted to elucidate some of these relationships.

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Materials and Methods

All experiments were conducted at the Friday Harbor Laboratory, Friday Harbor, Washington. Starfish (*Pisaster ochraceus*) were collected from the intertidal zone at various times during June and July and maintained in sea tanks (10°–13° C) until utilized. All experiments were conducted under *in vitro* conditions with sea water, filtered prior to use, being utilized as the incubation medium. The ten ovaries were removed from animals and placed in large finger bowls containing sea water. The finger bowls and assay dishes used in these experiments were placed in trays on a sea table in order to maintain a relatively constant temperature. The radial nerve factor (RNF) utilized in these experiments was lyophilized material obtained from nerves of *Asterias forbesi*, prepared as previously described (Schuetz and Biggers, 1968). A stock solution of RNF was prepared by dissolving 1 mg of the dried material in 1 ml of distilled water. This solution was then kept under refrigeration (4° C). Preliminary experiments demonstrated that RNF prepared from *Asterias forbesi* was effective in stimulating oocyte shedding, meiotic maturation, and formation of ovarian factor activity when added to ovarian fragments of *Pisaster ochraceus*. This RNF material did not produce meiotic maturation in isolated oocytes. Ovarian factor utilized in the present experiments was prepared from tissue of *Pisaster ochraceus* using methods previously described, with the exception that tissues were not usually squashed prior to the addition of RNF (Schuetz, 1969). A standard preparation of ovarian factor was used in all these experiments. This substance was separated from other components of the incubation medium by gel filtration.

Biological assays

The effects of the RNF and ovarian factor were evaluated by means of the weight change in isolated ovaries or ovarian fragments and the induction of meiotic maturation in isolated oocytes (Schuetz, 1967; Schuetz and Biggers, 1968). Prior to use, ovarian responsiveness to a standard preparation of RNF was assessed. Ovarian tissues responding with 60% or greater reduction in weight were used in these experiments. Ovarian fragments were obtained by cutting ovaries into approximately equal portions. The ovarian tissues were weighed, following blotting on absorbent paper, on a Mettler P160 balance to the nearest milligram. The ovarian tissue was then placed into finger bowls or small Stender dishes containing a standard amount of filtered sea water. At various times during or at the termination of the experimental treatment, the ovarian fragments were weighed following blotting. Ovarian fragment weight loss or oocyte shedding was expressed as the ratio of the final weight to the initial weight. The ratios presented for the various groups in the figures represent the mean ratio of all fragments in a particular group.

The effects of the various biological substances or extracts on the process of meiotic maturation were assessed using oocytes isolated from pretested ovarian tissue. Mincing ovaries with scissors resulted in the release of enormous numbers of oocytes each of which was surrounded by a single layer of follicular cells. Following manual removal of the ovarian tissues present in such a mince, the remaining free oocytes with intact germinal vesicles constituted the preparation

of "isolated oocytes." In all animals (> 50) studied during the summer the amount of "spontaneous" meiotic maturation in oocytes so released from ovaries was found to be less than 10%. The RNF preparation was ineffective in stimulating meiotic maturation in these oocytes. Following agitation, approximately 5000–10,000 oocytes were transferred to each assay dish containing a standard amount of filtered sea water. The test substances were then added to the dishes at varying times and for each the percentage of oocytes undergoing germinal vesicle breakdown was assessed. This was determined after agitating the dish and mixing all the oocytes. The number of oocytes with or without an intact germinal vesicle was determined within a restricted area with the aid of a dissecting microscope. A sample of at least 50 oocytes was normally examined.

Results

Time of ovarian factor appearance

In order to assess the time at which ovarian factor appeared in the incubation medium, the following experiment was performed. Four finger bowls were prepared with the following contents:

Bowl No.			
I.	1 ovary (13.7 gm)	+ 1.0 mg RNF	+ 100 ml filtered SW
II.	1 ovary (13.9 gm)		+ 100 ml filtered SW
III.		1.0 mg RNF	+ 100 ml filtered SW
IV.			100 ml filtered SW

At regular intervals following the addition of the RNF to the dishes and after agitation of the contents, a 10 ml sample of the incubation medium, free of oocytes, was removed by pipette and placed in a test tube. Shedding of oocytes became apparent approximately 70 minutes after the addition of RNF in Bowl I. The final weight of the ovary in Bowl I was 1.7 gm and in Bowl II, 13.4 gm. After all the 10 ml samples were collected, the presence of ovarian factor activity in the samples was assessed. Biological activity was determined by adding 0.5 ml from each sample collected to each of two test dishes containing free oocytes in 3.5 ml of filtered sea water. The mean results of ovarian factor activity expressed as the percentage germinal vesicle breakdown are presented in Figure 1. The data indicate that increased activity of ovarian factor was present in the media removed from the dish containing RNF and ovary together; whereas media containing only RNF or ovary were inactive in stimulating meiotic maturation. A marked change in activity of ovarian extract occurred after 100 minutes. Experiments similar to this one indicate that the time at which ovarian factor was first detected in the incubation media varied considerably and was dependent upon the amount of media tested.

RNF and ovarian factor induced shedding

The time at which oocyte shedding occurred in response to ovarian factor or to RNF were compared by recording the change in weight of ovarian fragments at varying times after addition of these two substances. Ovarian fragments were weighed and randomly placed into Stender dishes containing 4 ml of filtered sea

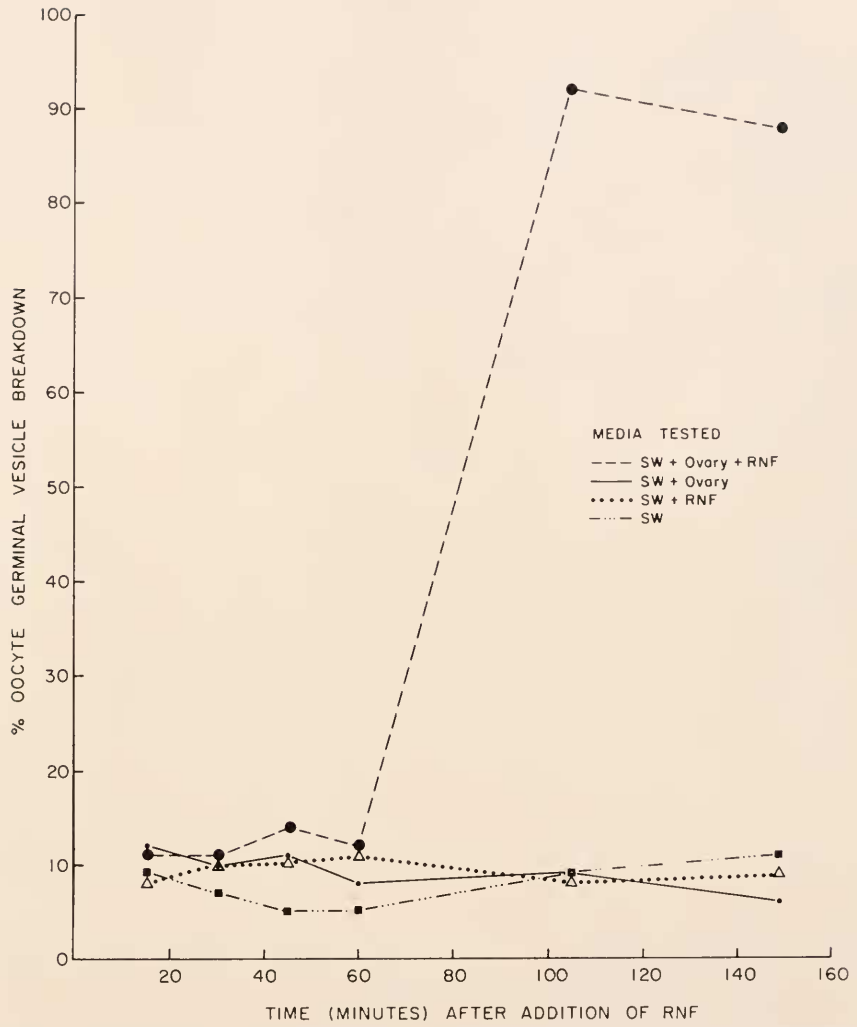

FIGURE 1. Appearance of ovarian factor activity in incubation media. Dishes of sea water were prepared with or without ovaries and with or without RNF added. Media were removed at various times and added to dishes containing isolated oocytes with intact germinal vesicles. Inducton of germinal vesicle breakdown (%) in isolated oocytes was assessed after a one hour exposure to the test media.

water. Varying amounts of ovarian factor (0.2, 0.6, 1.8 ml) or 100 μg of RNF were added to the different treatment groups. Each treatment group consisted of 5 fragments each in an individual dish. At varying times after the addition of these test substances, each fragment was removed, blotted, weighed, and then returned to the same dish from which it was taken. The sequential changes in weight of the variously treated fragments are presented in Figure 2. The data are presented as the mean ratio of the final weight to initial weight, but the final

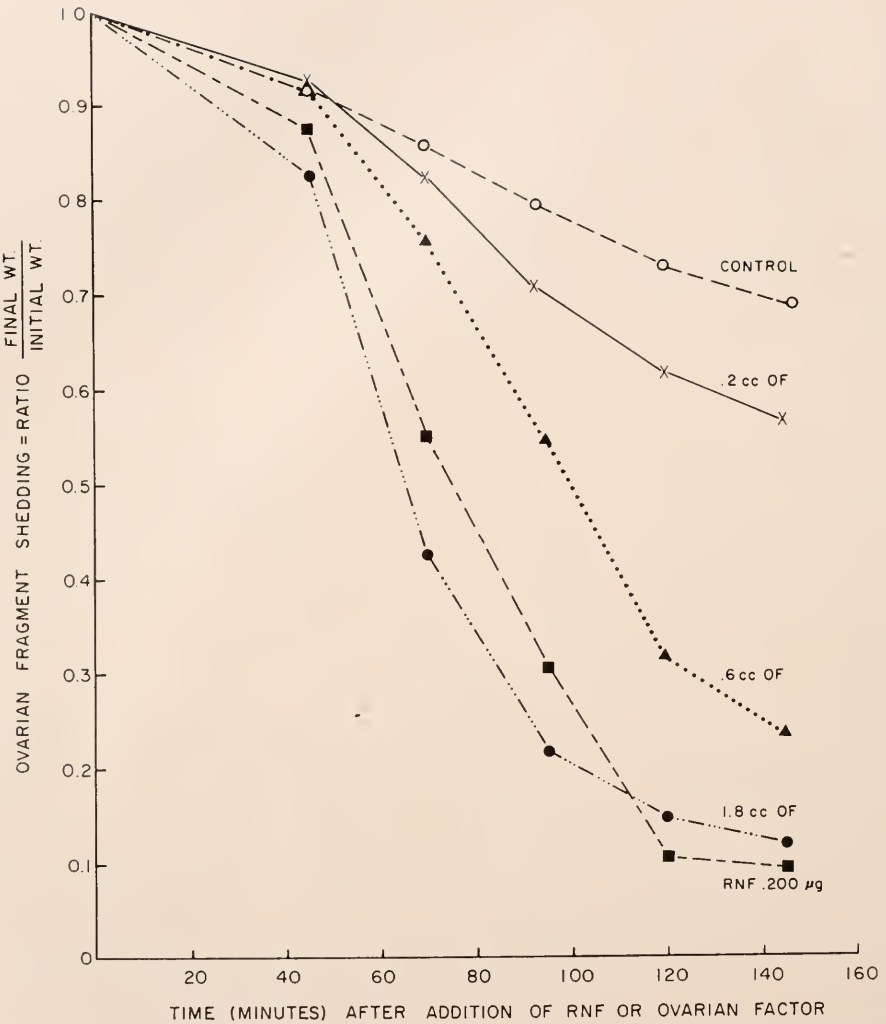

FIGURE 2. Ovarian fragment shedding—Effect of ovarian factor and RNF. Groups of five ovarian fragments were exposed to RNF or various concentrations of ovarian factor. The data represent the sequential change in weight of these fragments.

weight is in reality the weight of the fragment as measured at the designated times after the addition of RNF. These data indicate that little shedding occurred before 50 minutes after the test materials were added. The extent of shedding in response to ovarian factor was markedly dependent upon the dose added. Shedding occurred in a remarkably similar fashion in those fragments exposed either to the highest dose of ovarian factor or to RNF. Repetition of this experiment demonstrated the same type of finding; however, the time at which shedding was initiated varied in the different animals.

Effect of RNF exposure time on oocyte shedding

It is not known whether, or for how long, RNF is required to be in the presence of ovarian tissue in order for shedding to occur. This was tested by exposing groups of ovarian fragments to RNF for varying periods of time, washing them and returning them to fresh media containing no test material. Randomized groups of ovarian fragments were exposed to 100 μg of RNF in 15 ml of filtered sea water. One control group did not receive any RNF and one

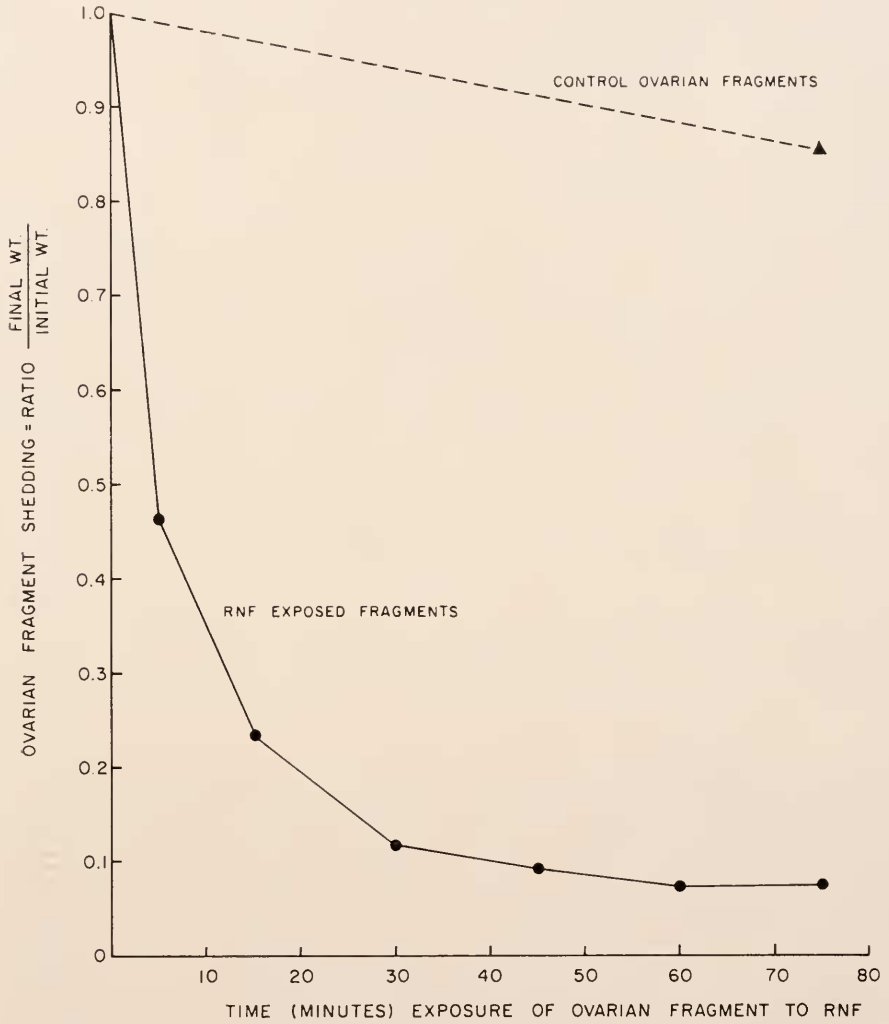

FIGURE 3. Ovarian fragment shedding—Effect of time of exposure to RNF. Groups of five ovarian fragments were exposed to RNF for various times (min). The tissues were washed and transferred to fresh media without RNF. Each dot represents the mean ratio of the five fragments.

group was exposed to RNF during the entire experimental period. After the designated period of exposure to RNF the fragments were removed, blotted and transferred through 3 washes (in a 6 minute period), each consisting of 300 ml of fresh sea water. Following washing fragments were placed in fresh sea water (15 ml). The final ovarian weight of all fragments was recorded 120 minutes after the fragments were originally exposed to the RNF. The results of this experiment are presented in Figure 3. A 5 minute exposure to RNF was sufficient to induce a 50% reduction in ovarian weight, whereas, a 30 minute exposure insured maximal shedding within the two hour test period. Shedding did not occur in control fragments incubated in the continual presence of RNF until after 60 minutes. Shedding appeared to occur sooner in those fragments exposed to RNF, and subsequently washed prior to being returned to RNF-free media, than in those exposed continuously to RNF.

Oocyte response to ovarian factor

The time at which germinal vesicle breakdown occurs in oocytes following exposure to ovarian factor and the length of time oocytes need to be exposed to ovarian factor are questions of importance. Free oocytes (5000–10,000) in 0.5 ml were added to 5 ml of filtered sea water and then 1 ml of the standard preparation of ovarian factor was added. Controls consisted of oocytes not exposed to ovarian factor or to RNF. Oocytes were incubated in the continual presence of ovarian factor or were exposed to ovarian factor for varying times and then transferred to fresh sea water containing no ovarian factor. Both parts of this experiment were performed using oocytes obtained from the same animal. The results of both aspects of this experiment are presented in Table I. In the first instance, 7 dishes containing oocytes were exposed to ovarian factor. At varying intervals after the addition of the ovarian factor, the percentage of oocytes with intact

TABLE I

Time of germinal vesicle breakdown in oocytes following exposure to ovarian factor (1 ml) and the effect of time of exposure of oocytes to ovarian factor (1 ml) germinal vesicle breakdown

Part I		Part II	
Time (min) after ovarian factor added to oocytes	% oocyte GVBD	Time (min) oocytes exposure to ovarian factor	% oocyte GVBD
0	6%	0	5%
5	7%	5	5%
10	9%	10	15%
15	10%	15	98%
20	12%	20	98%
25	25%	25	97%
30	85%	30	98%
35	98%	35	99%
Terminal control			
RNF (100 μg) treated oocytes	5%		7%

germinal vesicles in one dish was immediately recorded. The entire process of oocyte germinal vesicle breakdown occurred within a 3–5 minute period and was synchronous in all the oocytes released from a particular ovary. Germinal vesicle breakdown occurred in 98% of the oocytes 35 minutes after the addition of the ovarian factor.

For the second part of this experiment, 8 dishes of oocytes were exposed to ovarian factor and at subsequent 5 minute intervals one dish was flooded in turn with fresh sea water. This medium was poured off and the remaining oocytes were immediately transferred to a dish containing 100 ml of fresh sea water. Addition of 1 ml of ovarian factor to oocytes in 100 ml of sea water was ineffective in stimulating meiotic maturation. One hour after the initial exposure of oocytes to the ovarian factor, the percentage of germinal vesicle breakdown in all dishes was determined. Exposures of 5–10 minutes were insufficient to produce germinal vesicle breakdown, whereas, longer periods of time produced essentially 100% germinal vesicle breakdown. This breakdown occurred at essentially the same time as in those oocytes incubated in the continual presence of ovarian factor.

The time at which germinal vesicle breakdown occurred in isolated oocytes was also compared to the time of shedding from ovarian fragments exposed to RNF or ovarian factor. In each case these comparisons were made using free oocytes and ovarian fragments obtained from the same animal. These experiments, using tissue and oocytes from different animals, revealed in all cases that oocyte germinal vesicle breakdown induced with ovarian factor preceded shedding induced with either ovarian factor or RNF.

Discussion

Previous experiments have indicated that changes in the starfish ovary previously attributed to the actions of radial nerve factor (RNF) may also be produced by a possible intermediary substance called an ovarian factor (Schuetz and Biggers, 1967; Schuetz, 1969) or a meiosis-inducing substance (Kanatani and Shirai, 1967). In general, data presented here support this contention. The physiological changes studied were the processes of oocyte shedding and oocyte germinal vesicle breakdown.

Shedding of oocytes, following exposure of ovarian fragments to either RNF or ovarian factor, was remarkably similar in character and duration. The process of induced oocyte shedding, as reflected by the changes in the weight of the ovarian fragments, was divided into distinct phases (Fig. 2). Subsequent to the addition of RNF or ovarian factor, an initial lag phase occurred during which the weight of the ovarian fragment showed little change. In the data presented here, the duration of the lag phase was approximately 50 minutes. This initial phase was followed by a continuous decrease in the weight of ovarian fragments over a 50 minute period. In fragments exposed to ovarian factor the rate of weight change was markedly dependent upon the dose of ovarian factor present in the assay dishes. The rate and extent of weight loss in the ovarian fragment following exposure to either the RNF or the highest dose of ovarian factor were indistinguishable.

Previous investigators have described the presence of a lag phase for RNF-induced shedding (Chaet and Musick, 1960); however, the duration of the lag phase appears to vary considerably with the species and animal tested. Ovaries of *Pisaster ochraceus* exposed to RNF were consistently found to have a longer lag period than similarly treated ovaries of *Asterias forbesi* (Schuetz unpublished). Whether this is a true species difference or the result of the water temperature differences on the east and west coasts is not clear.

Although oocyte shedding and meiotic maturation resulted when ovarian fragments were exposed to RNF, meiotic maturation in isolated oocytes occurred only when exposed to ovarian factor (Fig. 2), and took approximately 30 minutes for completion (Table I). Thus, ovarian factor acted more rapidly in producing germinal vesicle breakdown in isolated oocytes (Table I) than in producing oocyte shedding (Fig. 2). This was true even when similar concentrations of ovarian factor were present in assay dishes containing oocytes and ovarian fragments. Thus, these data suggest that meiotic maturation, which occurs following ovarian tissue exposure to RNF, could be explained by an intermediate step since the time required for the ovarian substance to produce its effects was less than that required for the RNF. The question arises, however, of why its effect on the germinal vesicle in isolated oocytes (Table I) is more rapid than its effect on shedding of ovarian fragments (Fig. 2). Previous data demonstrated that the ovarian factor (Schuetz, 1969) and RNF (Kanatani, 1964; Schuetz, 1969) can act through the ovarian wall of ligated ovarian fragments to initiate meiotic maturation. A possible explanation is that the ovarian tissues are differentially permeable to one of these substances. Alternatively, if the RNF is producing or releasing ovarian factor from ovarian tissue, this ovarian factor will then be in closer proximity to the enclosed oocytes than the ovarian factor present in the incubation media.

In what cells or tissues the ovarian factor is found and whether it is released or synthesized in response to RNF is not at present clear. The time relationships described here (Fig. 3 Table I) indicate that RNF "activation" of ovarian factor is a relatively rapid process. It is not clear how the ovarian factor produced the changes in the ovarian fragments leading to oocyte shedding. Previous studies have indicated that disruption of the follicular cells from around oocytes may be a necessary prerequisite for shedding (Kanatani, 1964). The muscle tissues of the ovarian wall also appear to be important for the shedding response (Schuetz and Biggers, 1969; Kanatani, 1964; Macklenberg and Chaet, 1964). How these tissues and inductor substances interact to produce these changes is not yet clear. The induction of meiotic maturation in oocytes, however, appears to result from the direct action of the ovarian factor. The inability of the RNF to act directly on the isolated oocytes to produce germinal vesicle breakdown suggests that the oocyte is not itself the source of the ovarian factor. The possibility does exist, however, that the RNF, although not acting directly on isolated oocytes, could act on the non-shed oocytes causing them to release the ovarian factor. Kanatani *et al.* (1969) recently presented experimental evidence indicating that the meiosis inducing substance, which is comparable to the ovarian factor discussed here, is 1-methyladenine. The elucidation of the physiological and biochemical role of this substance should greatly enhance our understanding of the process of oogenesis.

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Summary

The addition of a radial nerve factor (RNF) to starfish ovarian tissue *in vitro* results in the release into the incubation media of a substance (ovarian factor) with different chemical properties but many biological properties similar to those of the RNF. A series of experiments were conducted to assess and compare the time course during which these substances initiate their biological effects. The effects of these substances on the process of oocyte shedding and germinal vesicle breakdown were assessed under *in vitro* conditions.

1. Within approximately a 1 hour period following the addition of RNF, ovarian factor activity was found in the incubation media. Activity in some instances was found prior to induction of shedding.

2. Oocyte shedding induced by the addition of both RNF and ovarian factor occurred folowing a lag period of approximately 1 hour. The rate of oocyte shedding in response to RNF and the highest dose of ovarian factor were indistinguishable.

3. Free oocytes exhibited germinal vesicle breakdown in response to ovarian factor but not to RNF. Germinal vesicle breakdown in response to ovarian factor occurred within 30 minutes of the addition of ovarian factor to free oocytes. Germinal vesicle breakdown in free oocytes occurred prior to either ovarian factor or RNF induced shedding from ovarian fragments.

4. The continual presence of RNF in the incubation media was not required for the induction of shedding from ovarian fragments.

5. Free oocytes did not require the continual presence of the ovarian factor for germinal vesicle breakdown to occur. An exposure time of approximately 15 minutes insured meiotic maturation in free oocytes.

These data support the hypothesis that the ovarian factor is a normal intermediary substance in RNF-induced shedding and in oocyte germinal vesicle breakdown or meiotic maturation.

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